Investigation of bee mortality in a beekeeper operation in the Coromandel district

Introduction

The Ministry for Primary Industries (MPI) was notified during October 2014 of a bee mortality event in a beekeeper operation in the Coromandel district. Early in the ensuing investigation a Plant and Food Research scientist concluded that the event described did not fit with known exotic causes of bee mortality (European foulbrood and bee tracheal mite). This was confirmed by an Apiary Officer (AO) but after interviewing the beekeeper the AO determined that the impact of the event was sufficient to warrant further investigation.

None of the major exotic bee pathogens were detected but the trypanosome *Lotmaria passim* was identified. (Until recently this was classified incorrectly as *Crithida mellificae*, a honey bee parasite not previously detected in New Zealand.) First found in Australia in the 1960s, it has only recently been suggested to be associated with winter losses in bees. The present investigation did not show an association with bee losses, but only a few colonies were tested. There was no correlation found for the level of agents *L. passim* and *N. ceranae* present in affected colonies, but these analyses do not exclude that possibility. Testing live bees remaining in the affected colony, rather than the bees that had left, may have concealed a true association. In addition, the levels of these agents at the time that losses are occurring is not necessarily relevant. Reports in the literature show the levels of these agents in summer are associated with bee losses occurring in winter.

L. passim has subsequently been identified in beekeeper operations outside of the Coromandel district, suggesting it could have been here for some time but only detected recently through new molecular diagnostic methods.

Background

The causes of bee mortality can be challenging to investigate because it is impossible to assess the significance of all the potential agents found. Interactions among these agents can make the causal web of disease very complex. In addition, clinical signs of disease in bees tend to be so generalised that interpreting clinical signs and their significance in a specific colony can be problematic. There are also limited diagnostic methods for understanding disease in bees. Where multiple mortality events occur within separate beekeeper operations it can be hard to tell whether these are related to the same event or unrelated events. Multiple beekeeper operations are affected by diverse factors including climate, varroa infection and feed availability.

To address these difficulties, MPI established a working group comprising several parties, including the affected beekeeper, AsureQuality, Plant and Food Research, and other known bee experts.

Initial investigations

As part of the investigation, a bee scientist made an initial visit to the beekeeper operation and examined bees and colonies from five affected apiaries. Generally the hives had sufficient honey and most had stored pollen reserves. There was evidence of endemic disease but no indication that infection levels were higher than normal. Several cells in one hive had chalk brood; three hives had larval clinical signs consistent with sacbrood virus in unused comb; and two bees were seen with deformed wings possibly caused by deformed wing virus (DWV). There was no indication of clinical American foulbrood (AFB); nor were varroa or small hive beetle (both exotic to New Zealand) observed. All colonies examined had laying queens.

In the hives from affected apiaries, bee numbers had declined from an estimate of 10 000–20 000 to 1000–2000 over a period of one month and the brood area had shrunk from 4–5 frames to about half a frame. There were large numbers of eggs present, compared with the number of larvae and capped cells. Although no measurements were taken, there appeared to be very low conversion rates from eggs to pupae: possibly as low as 5 percent, when 80–100 percent would be expected. A further observation was that there was little larval food with the larvae. There did not appear to be any health issue with the queen where it had been removed from the weak hives and placed in unaffected colonies (n = 10). When five of these colonies were combined with healthy colonies, with a queen excluder between them, they quickly recovered without further heath issues.

After investigation had commenced on this beekeeping operation, it was noticed that similar effects had been noted by five other commercial beekeepers on the Coromandel Peninsula.

Although this presentation did not fit with that of known exotic agents, bee samples were collected during October 2014 from 10 of the worst-affected colonies and tested. Samples were negative by PCR for European foulbrood (EFB), and tracheal mite. In addition, quantitative PCR (qPCR) was used to determine the levels of DWV, black queen cell virus (BQCV), chronic bee paralysis virus (CBPV) and members of the Dicistroviridae family (DF). This family includes Kashmir bee virus (KBV), acute paralysis and Israeli-associated paralysis viruses (IAPVs); IAPVs are not known to be present in New Zealand (McFadden *et al.* 2014). Tests were negative by qPCR for CBPV but positive for KBV (confirmed by DNA sequencing). Samples had only low to moderate levels of DWV and BQCV, suggesting these were not major pathogens in affected colonies. However, when interpreting these results we need to be aware that bees making up the high percentage that deserted their colonies could not be tested; furthermore, only live bees were tested.

Investigations of the significance of *Nosema*

Spore counts were determined for *Nosema* spp. and some of these counts were extremely high: about 100 million per bee. Quantitative PCR was used to confirm these levels and differentiate between counts for *N. apis* and *N. ceranae*. In order to understand the significance of this infection, in mid-

December 2014 surviving bees were collected and tested from 33 hives in a number of apiaries within the beekeeper operation. Samples were grouped by colony status, which was based on observed clinical signs, each group being categorised as "affected", "unaffected" or "unknown". Samples were also collected from 10 unaffected colonies in another beekeeper operation in the same general area; while these were considered to be "negative controls", all colonies in the Coromandel area could have been affected.

Nosema tests were generally positive for both *N. apis* and *N. ceranae* regardless of colony status (**Figures 1 & 2**). Linear regression was used to compare the PCR Cq values (amount of genetic material of the agent: typically, the lower the Cq number the more target organisms present in the bees), based on group status, with Cq normalised by natural logarithm transformation. The groups within the affected beekeeper operation did not have significantly higher *N. apis* levels than the negative control group. *N. ceranae* levels were greater in the affected beekeeper operation than the negative control group ($p < 0.01$) but not significantly different between status groups within the affected beekeeper operation.

Logistic regression was carried out to identify interaction between the levels of *N. apis* and *N. ceranae*, with the response variable coded dichotomously as affected or unaffected; the latter category was expanded to include "negative controls". There was no significant interaction between status and the Cq of *N. apis* and *N. ceranae*.

While the study of *Nosema* was not sufficiently comprehensive to answer all questions, data showed that the presence of the syndrome (based on beekeeper assessment of colony status) in the affected beekeeping operation was not associated with the levels of *Nosema* spp. at the time of testing. It is possible that there was a lag between measurement of *Nosema* spp. by qPCR and the development of clinical signs observed. If this were the case "unaffected" and "unknowns" could have had the agents present but no clinical signs at the time of sampling. If so, a true association may not have been detected because of the timing of sampling.

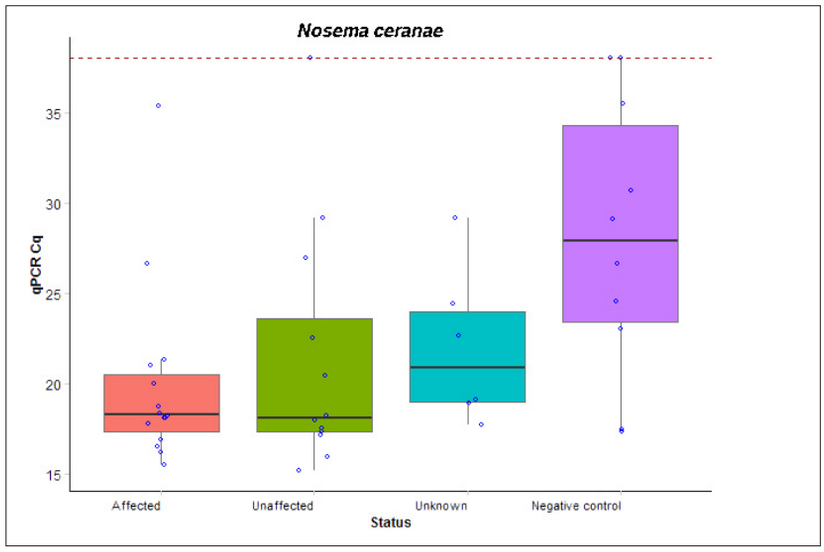

Figure 1

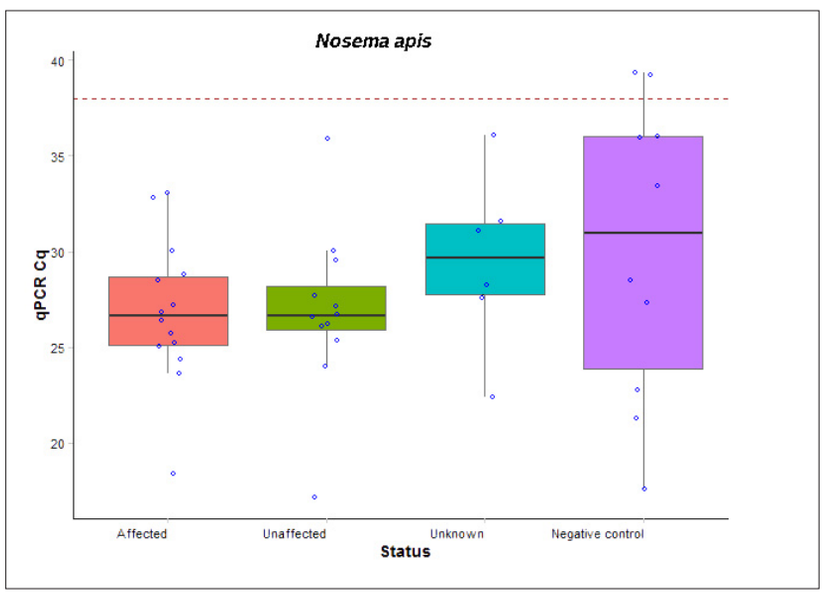

Figure 2

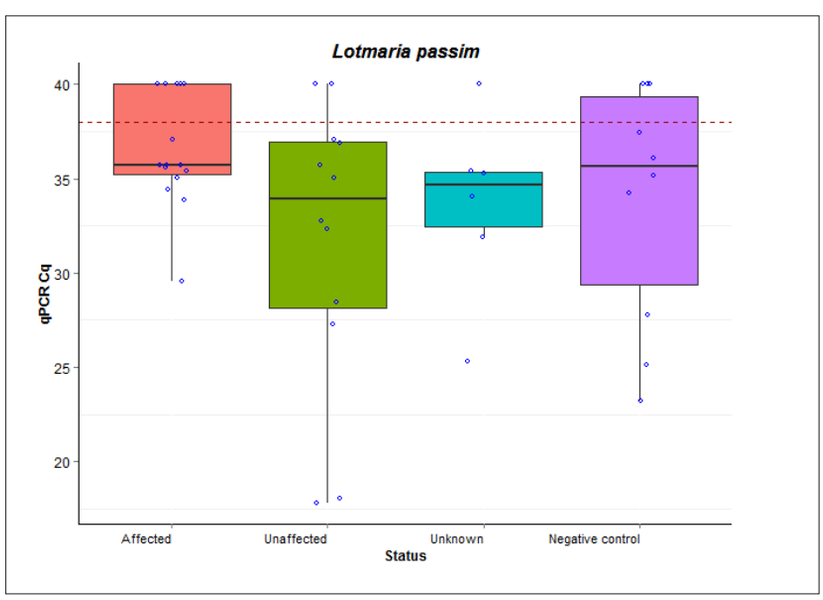

Figure 3

Boxplots of the amount of genetic material for a number of bee pathogens: *Nosema ceranae* (Figure 1), *Nosema apis* (Figure 2) and *Lotmaria passim* (Figure 3) present in bee samples collected from colonies classified as "affected", "unaffected", "unknown" and as a "negative control". The higher the Cq value the less the amount of the target organism present in the bee samples. A box plot provides the minimum, maximum and the interquartile range of Cq values. The negative control consisted of colonies from apiaries from another beekeeper that were not affected.

There is insufficient information to interpret the difference in *N. ceranae* levels between the affected beekeeing operation and the negative control colonies; although the levels were different, the negative controls were still generally strongly positive. The R^2 value for the regression was 16 percent, indicating that a number of other factors other than *N. ceranae* levels must have explained the difference in colony status.

Testing for other agents

Further testing was carried out for other possible agents, based on the hypothesis that the cause of the disease was multifactorial. This investigation centred around two agents that have been associated with winter bee losses and where a synergistic effect with *N. ceranae* has been reported. These were the insecticide fiprinol (Aufauvre *et al.,* 2012) and the trypanosomid parasite *L. passim* (*Crithida mellificae*; Ravoet *et al.,* 2013). Fiprinol was not detected in toxicological tests at Hills Laboratories, Hamilton.

According to Schwarz *et al.* 2015, reports of *C. mellificae* in honey bees are the result of misclassification and the species is in fact *Lotmaria passim,* which is globally the predominant trypanosome parasite of honey bees. This species has been known in Australian bees since 1967, but has only recently been implicated as pathogenic. It has been detected in many countries including North America, Asia and Europe. There have been no reports in New Zealand, but until recently no PCR assay was available, so detection does not necessarily signify a new incursion.

The impact of *N. ceranae* in winter increases with levels of *L. passim* (*C. mellificae*) and *N. ceranae* in summer (Ravoet *et al.,* 2013). Ravoet *et al.* (2013) speculated that variable impact of *N. ceranae* may be the result of factors such as *L. passim*. A related agent, *Crithidia bombi,* has for some years been known to infect bumble bees (Brown *et al.,* 2000).

The same 43 colonies that had been used to examine the relationship between *Nosema* spp. and colony status were tested for the presence of *L. passim*. Of these, 74 percent were positive for *L. passim* although in most cases there was a high Cq value, i.e., a low level of target organisms in the bees (**Figure 3**). Linear regression was again used to compare the Cq values between colony groups. The Cq (log transformed) of the "unaffected" colonies was lower (higher amount of target organism in the bees) than that for other groups. Thus higher levels of *L. passim* at the time of testing were not associated with disease. Logistic regression was carried out to identify interaction between the levels of *N. ceranae* and *L. passim* and colony status. No interaction was observed using this analysis.

Conclusions

Further research is necessary to understand the significance of mortality events such as this, using a longitudinal design of multiple beekeeper operations. To understand the complex interactions between agents and the effect of the lag between exposure and development of clinical signs would require multiple tests over several seasons.

Landcare Research, in conjunction with the bee industry and MPI, is carrying out a survey of 250 of the biggest beekeeper operations in New Zealand to find out more about colony losses over the past 12 months. Further work will also be carried out to determine what bee pathogens are present. A key issue is establishing a baseline of what level of bee losses is normal, and determining the associated risk factors in beekeeper operations where mortalities occur. Apiary samples will be tested for exotic bee diseases and the extent to which the bees are infested with endemic *Varroa* mites and *Nosema* spores. This research will be conducted in 2016.

ACKNOWLEDGEMENTS

The authors would like to thank other members of the working group and those who have assisted with the investigation: Simon McDonald, Byron Taylor, Jenny Draper, Grant Munro, Ric Clough, Lou Gallagher and Howard Pharo.

REFERENCES

Aufauvre J, Biron DG, Vidau C, Fontbonne R, Roudel M, Diogon M, Vigue`s B, Belzunces LP, Delbac F, Blot N (2012). Parasite-insecticide interactions: a case study of *Nosema ceranae* and fipronil synergy on honey bee. *Scientific Reports* 2, 326, 10.1038/srep00326. Accessed 15 November 2015.

Brown MJF, Loosli R, Schmid-Hempel P (2000). Condition-dependent expression of virulence in a trypanosome infecting bumblebees. *Oikos* 91, 421–427.

McFadden AMJ, Tham K, Stevenson M, Goodwin M, Pharo H, Taylor B, Munro G, Owen K, Peacock L, Stone M (2014). A national survey for proof of freedom of Israeli acute paralysis virus in *Apis mellifera* in New Zealand. *Journal of Apicultural Research* 53(5), 520–527, http://dx.doi.org/10.3896/IBRA.1.53.5.03. Accessed 15 November 2015.

Ravoet J, Maharramov J, Meeus I, De Smet L, Wenseleers T, Smagghe G, de Graaf DC (2013). Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. *PLOS ONE* 8, 10.1371/journal.pone.0072443. Accessed 15 November 2015.

Runckel C, DeRisi J, Flenniken ML (2014). A Draft Genome of the Honey Bee Trypanosomatid Parasite *Crithidiamellificae. PLOS ONE 9*, 10.1371/journal.pone.0095057. Accessed 15 November 2015.

Schwarz RS, Bauchan GR, Murphy CA, Ravoet J, de Graaf DC, Evans JD (2015). Characterization of two species of Trypanosomatidae from the honey bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, 1967 and *Lotmaria passim* n. gen., n. sp. *Journal of Eukaryote Microbiology*. doi: 10.1111/jeu.12209. Accessed 15 November 2015.

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